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An Enzyme(s) That Converts Glutaminyl-peptides into Pyroglutamyl-peptides

PRESENCE IN PITUITARY, BRAIN, ADRENAL MEDULLA, AND LYMPHOCYTES*

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The mechanism for the post-translational conversion of glutamine to pyroglutamic acid on the N terminus of newly synthesized peptides and proteins is unknown. An assay is reported that permits measurement of the rate of conversion of Gln-His-Pro-NH_2 to <Glu-His-Pro-NH_2 (TRH). Using this assay, we demonstrate that the spontaneous cyclization of the N-terminal glutamine of this peptide occurs only slowly under physiological conditions. Furthermore, we describe the presence in rat brain, porcine pituitary, and human B lymphocytes of an enzyme(s) which converts Gln-His-Pro-NH_2 into <Glu-His-Pro-NH_2. The enzyme(s) appears to be a glycoprotein, is maximally active at neutral pH, has a M_r of 55,000, and contains catalytically significant sulfhydryl groups. The product of the enzymatic reaction was confirmed by high resolution fast atom bombardment-mass spectrometry.

In preliminary studies, we find that over 90% of the enzyme in bovine adrenal medulla is contained in the soluble chromaffin vesicle fraction. These findings indicate that *in vivo* the post-translational conversion of a glutaminyl-peptide into a pyroglutamyl-peptide is neither spontaneous nor abiotic as has been previously proposed.

The N-terminal amino acid of many proteins, hormones, and neurotransmitter peptides is pyroglutamic acid (5-oxo-L-proline). Thyrotropin releasing hormone (TRH, <Glu-His-Pro-NH_2),[1] luteinizing hormone releasing hormone (<Glu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2), gastrin, and the heavy chain of γ-globulin are examples. At present, the mechanisms by which pyroglutamic acid is inserted onto the N terminus of such proteins and peptides are unknown. For example, pyroglutamic acid can neither be incorporated into tRNA (1) nor inserted directly into a growing peptide (2, 3). Although extracts of papaya latex can enzymatically cyclize Gln-tRNA to <Glu-tRNA (EC 2.3.2.5) (4), <Glu does not possess a reactive amine and, therefore, could occupy only the amino terminus of a nascent peptide chain, a position reserved for *N*-formylmethionine and the signal sequence in secretory proteins (5). Thus, for these several reasons and because nearly all secretory peptides and proteins are synthesized as part of a larger precursor, an N-terminal pyroglutamyl moiety is believed to result from the post-translational modification of either glutamine or glutamic acid. Furthermore, analysis of the structure of the genes for TRH (6, 7), leuteinizing hormone releasing hormone (8), and the heavy chain of γ-globulin (9) suggests that glutamine, and not glutamate, is the amino acid antecedent of the N-terminal pyroglutamic acid. In 1963, Messer and Ottesen (10, 11) suggested the presence of an enzyme in crude papain that deamidated either glutamine or glutaminyl peptides to <Glu-peptides (EC 2.3.2.5). Over the years, however, because of methodological difficulties in studying relatively unstable glutaminyl-peptides, mammalian enzymes catalyzing this important post-translational modification have not yet been identified (1), and the existence of such an enzyme has been questioned. This has led to the postulate that the post-translational cyclization of an N-terminal glutamine is spontaneous and abiotic (6). We now present evidence that the spontaneous cyclization of an N-terminal glutamine occurs only slowly under physiological conditions. We also demonstrate the presence in rat brain, porcine pituitary, bovine adrenal medulla, and human B lymphocytes of enzyme(s) which convert Gln-peptides into <Glu-peptides.

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[1] The abbreviations used are: TRH, thyrotropin-releasing hormone; MOPS, 4-morpholinepropanesulfonic acid; MES, 4-morpholineethanesulfonic acid; RIA, radioimmunoassay; tBOC, *t*-butoxycarbonyl.

MATERIALS AND METHODS

Assay for Glutamine Cyclase—An assay for glutamine cyclase activity was developed based on the substrate Gln-His-Pro-NH_2. Conversion of this substrate to the product, <Glu-His-Pro-NH_2, was measured by radioimmunoassay using an antiserum raised in New Zealand White rabbits against the immunogen <Glu-His-Gly-COOH (Penninsula Labs, Belmont, CA) coupled through its carboxyl terminus to bovine serum albumin at a molar ratio of 20:1 by means of the water-soluble carbodiimide, 1-ethyl-3-3-dimethylaminopropylcarbodiimide·HCl. Immunization was by the multiple intradermal method of Vitakaitis *et al.* (12). A radioimmunoassay was developed from this antiserum using ^{125}I-TRH as tracer, TRH (Penninsula Labs, Belmont, CA) as standard, phosphate-buffered saline, pH 7.5, and an assay protocol as described elsewhere (13). The within assay and between assay coefficient of variation was 6.1 and 6.7%, respectively, as determined by the method of Rodbard (14).

α-N-tBoc-Gln-His-Pro-NH_2 was synthesized by standard techniques using dicyclohexylcarbodiimide, and its purity and amino acid content verified as previously described (15, 16). Gln-His-Pro-NH_2 was freshly prepared as needed by reaction of the α-N-tBoc-Gln-His-Pro-NH_2 with 3.5 M HCl in anhydrous dioxane, followed by drying under a stream of nitrogen and dissolution of the product in 50 mM

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MOPS, pH 7.2. The Gln-His-Pro-NH_2, stored in this manner at −20 °C, is stable for 2–3 weeks.

Fifty μl of enzyme sample in 50 mM MOPS, pH 7.2, were added to small glass tubes followed by the addition of 30 μl of a mixture containing 10 nmol of Gln-His-Pro-NH_2 and other additives for testing. The samples were covered and incubated for 2 h at 37 °C in a shaking water bath. Blank assays contained boiled tissue samples as well as the tested additives. In addition, samples containing active enzyme were also incubated without substrate to verify that the product resulted solely from the conversion of Gln-His-Pro-NH_2 and not from an endogenous precursor. The reaction was stopped with 2 ml of ice-cold water and 10-μl aliquots removed and lyophilized for assay of <Gln-His-Pro-NH_2 as described above. For determination of the pH optimum, enzyme fractions were dialyzed against a panel of buffers each of which contained 50 mM MOPS, 50 mM MES, and 50 mM Tris, and previously adjusted to the desired pH.

Preparation of Enzyme—Eighteen g of porcine pituitary (Pel-Freeze, Rogers, AK) were homogenized in 40 ml of 50 mM MOPS, pH 7.2, frozen, and thawed twice, and further disrupted with a sonic probe. The homogenates were centrifuged at $4 \times 10^4 \times g$ for 30 min. Forty ml of the supernatant were applied to a column of Sephadex G-100 (4.5 × 100 cm) and eluted with 200 mM NaCl, 50 mM MOPS, pH 7.2, into 15-ml fractions. Twenty-five ml (27.8 mg) of fractions 25–42 from the Sephadex G-100 chromatography were loaded onto a DEAE-cellulose column (2 × 12 cm) and eluted with 50 mM MOPS, pH 7.2, and increasing concentrations of NaCl. Five-ml fractions were collected and dialyzed against 40 mM MOPS, pH 7.2.

Isoelectric focusing in agarose-Sephadex was by the method of Manrique and Lasky (17). Hydrogen ion concentration was measured by a surface electrode. Fractions were cut from the slab and centrifuged at 32,000 × *g* to "wring out" the gel.

For all purification steps, protein was measured by the method of Lowry *et al.* (18), and 50-μl aliquots were assayed for glutamine cyclase activity as described above. Enzyme from neonatal rat brain and human B lymphocyte cell lines P3X63Ag8 (secreting) and P3X63Ag8 (nonsecreting) was prepared in an identical manner with the exception that isoelectric focusing was not performed.

Affinity Chromatography—To columns containing 2–3 ml of concanavalin A, lentil lectin, or uncoupled Ultrogel support (LKB, Gaithersburg, MD) were added 1-ml aliquots of the G-100 enzyme preparations. The columns were washed with 4 column volumes of 200 mM NaCl, 50 mM MOPS, pH 7.2, and then with 5 volumes of the same buffer containing either 0.2 M α-D-glucopyranoside or 0.2 M α-D-mannopyranoside. One-ml fractions were collected and analyzed for glutamine cyclase activity as outlined above. Neither 0.2 M D-Glucopyranoside nor 0.2 M D-mannopyranoside inhibited the enzyme when added directly to the assay.

Affinity chromatography over 2–3-ml columns of organomercurial-agarose (Bio-Rad) was carried out in a similar manner except that the columns were eluted with a gradient of 0–200 mM dithiothreitol and the fractions dialyzed before assay against the saline/MOPS buffer without dithiothreitol. Control experiments indicated that 85% of the enzyme activity could be recovered after incubation with dithiothreitol and dialysis.

Verification of Product—The specificity of the RIA for product was verified by determining the affinity of the antisera for authentic TRH and a number of congeners including <Glu-His-Pro-COOH, <Glu-His, His-Pro-diketopiperazine, Glu-His-Pro-NH_2, and the substrate, Gln-His-Pro-NH_2. To verify the identity of the product, 5.62 mg (5 ml) of the G-100 effluent were incubated with 1.65 μmol of Gln-His-Pro-NH_2. The reaction was stopped with 100 ml of ice-cold water and immediately applied at 4 °C to a column (1.5 × 12 cm) of Dowex 50 (cationic, 4% cross-linked) previously equilibrated with 0.05 M sodium citrate, pH 6.8. The effluent was monitored by TRH RIA, and fractions containing immunoreactive TRH were pooled. The pooled samples were charcoal extracted and purified by reverse-phase high performance liquid chromatography using a pyridine/acetic acid/propanol mobile phase, as previously described in detail (19). Immunoreactive fractions were again pooled, lyophilized, and submitted for mass spectrometry or subjected to TLC. Blanks were carried through the same purification steps, and recovery of product and possible chemical generation of additional product monitored throughout by use of a classical and well characterized RIA for TRH (13). For TLC, plates were developed in CH_3OH, $CHCl_3$, 38% CH_3COOH (40:60:20) and treated with iodine vapor and the Pauly reagent.

Mass spectrometry was performed on a VG 7070 E-Q tandem, high resolution instrument (VG Instruments, Stamford, CT) fitted with a fast atom bombardment system supplied by the manufacturer. Approximately 5 μg of standard or sample were placed on the target with thioglycerol (10 μl). The target was bombarded by xenon atoms at 8 kEV translational energy while the positively charged secondary ions were detected by scanning the mass spectrometer cyclically over the mass range 500–50 daltons at 5-s intervals. Accurate mass measurements were performed by peak matching at a resolution of 5000 (10% valley definition) using selected acylcarnitines as reference mass standards.

RESULTS AND DISCUSSION

The antiserum developed in this study has an affinity for TRH (2.2×10^7 M^{-1}) over 10^2-fold higher than for the substrate, Gln-His-Pro-NH_2, and 20-fold higher than for the expected product of a glutaminase, Gln-His-Pro-NH_2 (1.16×10^6 M^{-1}). The antibody does not recognize His-Pro-diketopiperazine ($k_{aff} < 10^3$ M^{-1}) and has an affinity for <Glu-His of only 4.4×10^5 M^{-1}. Use of this antibody permits measurement of the product, TRH, in the presence of substrate and avoids the need for physical separation of Gln-His-Pro-NH_2 and TRH that can lead to the spontaneous cyclization of Gln-peptides (20).

Incubation of Gln-His-Pro-NH_2 with fractions of porcine pituitary obtained by gel chromatography identified a region (fractions 19–42) that converted the substrate into TRH (Fig. 1). The enzyme appeared to have a M_r of 55,000 as determined by co-chromatography with standards, and its activity was maximal at a pH of approximately 7.2–7.5 with a rapid loss

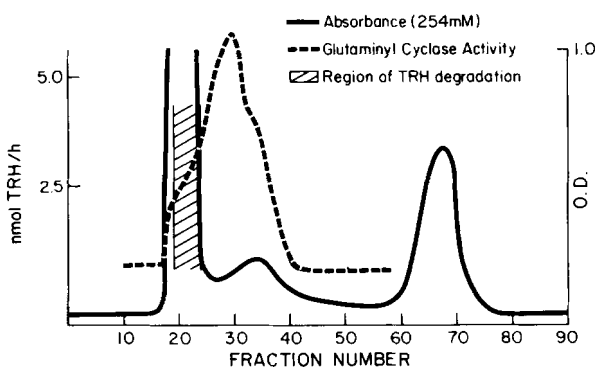

FIG. 1. **Formation of <Glu-His-Pro-NH_2 (TRH) from Gln-His-Pro-NH_2 by fractions obtained from gel permeation chromatography of porcine pituitary.** In this and other figures, see "Materials and Methods" for details.

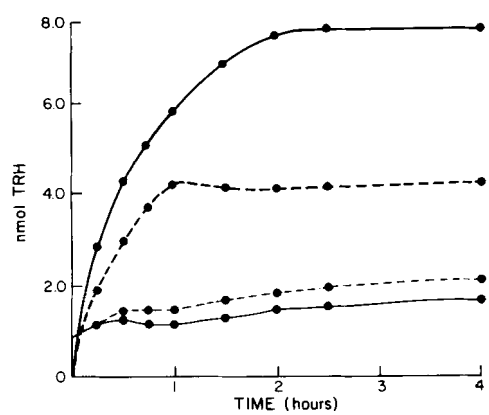

FIG. 2. **Time course for the formation of <Glu-His-Pro-NH_2 (TRH) from Gln-His-Pro-NH_2 in the presence (——) and absence (– – –) of 50 mM EDTA by 50 μl of a pool of fractions 25–42 from the gel permeation chromatography shown in Fig. 1.** Formation of TRH by active aliquots is represented by the *higher set* of two *curves* (blank values subtracted), and that formed by boiled aliquots (blanks) is represented by the *lower set* of two *curves*.

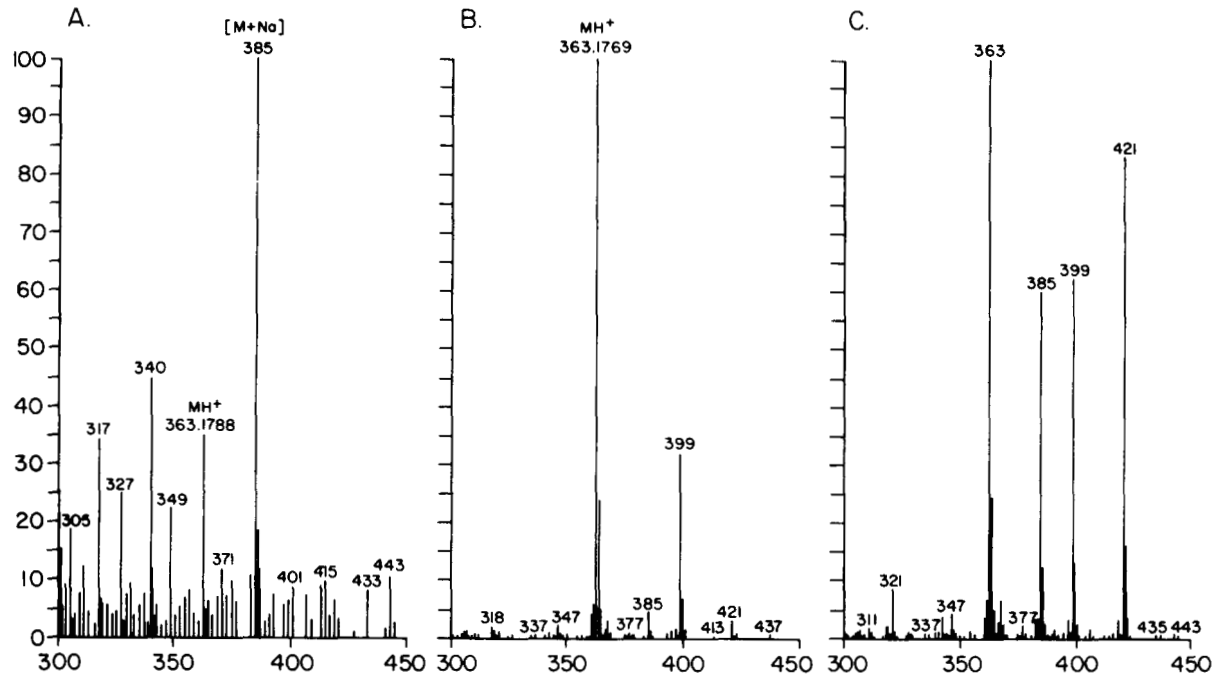

FIG. 3. **Fast atom bombardment-mass spectra of the purified product of the glutaminyl-cyclase (*a*), TRH standard (*b*), and TRH standard + Na thioglycerol (*c*).**

of activity above pH 8.0 or less than 5.0. The first several fractions of cyclizing activity (19–24) also contained TRH degrading enzymes as well. No fraction formed TRH in the absence of added substrate, verifying that TRH was formed only from Gln-His-Pro-NH_2 and not from an endogenous precursor.

The formation of <Glu-His-Pro-NH_2 by a pool of fractions 25–42 from the gel chromatography, containing 1.1 mg/ml protein and excluding the TRH degradative activity, as compared with boiled samples of the same pool is shown in Fig. 2. In the boiled tissue blanks at time zero, there is a small amount of TRH immunoreactivity present, representing the minor cross-reactivity between the substrate, Gln-His-Pro-NH_2, and the antibody used to measure product. Over the course of 4 h, however, there is a very slow, but measurable conversion of the substrate into authentic TRH (0.07 nmol/h, K (rate constant) = 0.008 h^{-1}) in the absence of active enzyme. In the presence of the active enzyme, however, the initial rate of production of TRH (active preparation − blank values) is 160-fold greater, 11.4 nmol/h (K = 1.33 h^{-1}), a rate enhanced 2-fold by the addition of 10 mM EDTA. Thus, in the presence of 10 mM EDTA, 50 μl (0.055 mg) of the G-100 pool exhausted 10 nmol of substrate in 2 h, converting nearly 80% into TRH.

The product was verified by three methods: 1) immunological, 2) TLC, and 3) mass spectometry. After 3 h, assay of the product from an incubate containing 5.62 mg of the G-100 extract yielded 309 μg of TRH immunoreactivity while the blank contained 27.5 μg. Following Dowex chromatography, charcoal extraction, and high performance liquid chromatography of the products, total recovery of TRH from the experimental sample was 270 μg when assayed by either the N-terminal TRH RIA described above or by classical TRH RIA (13). Because of the initial rapid removal of Gln-His-Pro-NH_2 by the Dowex at 4 °C, no significant generation of product was observed in the samples during purification. Furthermore, the purified product migrated as a single, Pauly positive spot on silica gel TLC with an R_F identical to that of an authentic TRH standard.

Fast atom bombardment-mass spectra of the purified product were characterized by a protonated molecular ion (MH^+) at m/z 363 and an intense sodium adduct ($[M + Na]^+$) at m/z 385 (Fig. 3*A*). A standard of authentic TRH gave an intense MH^+ ion at 363 (Fig. 3*B*). Reanalysis of the TRH standard in sodium thioglycerol caused the appearance of an intense sodium adduct at m/z 385 (Fig. 3*C*). By peak matching, the accurate mass of the m/z 363 ion in the sample was determined to be 363.1788 ($C_{16}H_{23}N_6O_4$ requires 363.1776) while that of authentic TRH was 363.1769. The single Pauly positive spot co-migrating with standard by TLC, the negligible deviation of the measured mass from the required mass and the common fast atom bombardment-generated MH^+ and $[M + Na]^+$ ions in sample and standard strongly support the identity of the product as TRH.

Further purification of the Gln-cyclizing preparation from porcine pituitary on DEAE-cellulose produced two major peaks of enzyme activity (Fig. 4), the first (fractions 18–21, 0.17 mg of protein/ml) with a K_m for Gln-His-Pro-NH_2 of 0.2 mM and a specific activity (V_{max}) of 2.3 nmol/μg/h; and the second (fractions 27–31, 0.28 mg of protein/ml), a K_m of 0.6 mM and a specific activity (V_{max}) of 1.4 nmol/μg/h. Both peaks of activity obtained from the DEAE chromatography bound tightly to concanavalin A, lentil lectin, and organo-

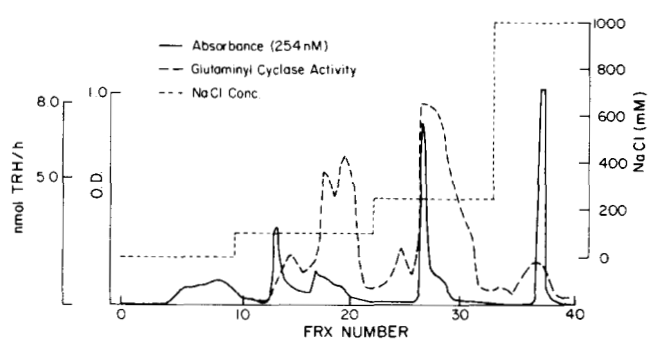

FIG. 4. **Further purification of cyclizing enzyme from porcine pituitary by DEAE-cellulose chromatography.**

mercurial-agarose, suggesting the presence of sulfhydryls and *N*-linked, glycosidic side chains.

Cofactor requirements of the two DEAE peaks are very similar, both being inhibited by transition metals and *N*-ethylmaleimide, and stimulated by NaCl and EDTA (Fig. 5). The second peak, however, appears to be partially inhibited by dithiothreitol; phenylmethylsulfonyl fluoride; and diethyldithiocarbamate whereas the first peak of activity is not. Interestingly, both are partially inhibited by FADH and completely inhibited by reduced 6-methylpterin and 2 mM 1,10-orthophenanthroline (data for orthophenanthroline not shown). The inhibition of the enzyme by 1,10-orthophenanthroline (a planar heterocyclic that coordinates many planar transition metals) contrasts with the stimulation by EDTA. The reasons for this inhibition are unclear, but likely relate to structural features that are independent of orthophenanthroline's metal coordinating properties. Whether the minor differences observed between the two peaks of catalytic activity reflect the presence of two distinct enzymes, different chemical forms of the same enzyme (*e.g.* differing degrees of glycosylation), or the presence of confounding impurities will require further study.

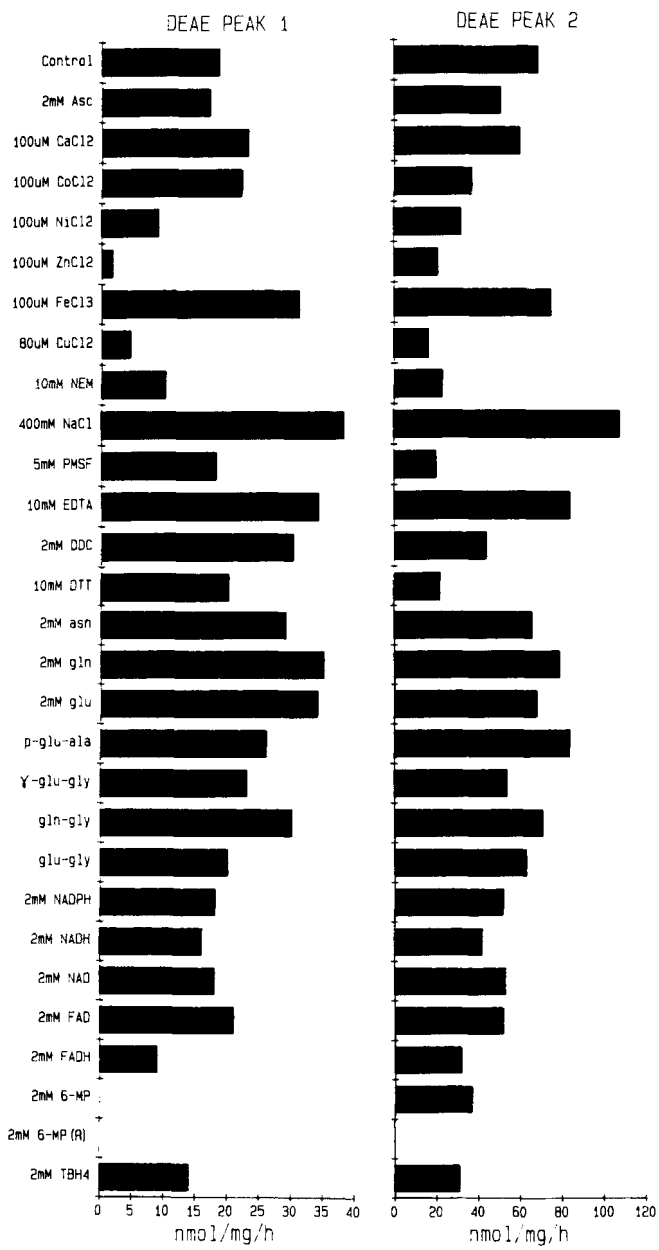

FIG. 5. **Comparison of catalytic properties of the two major peaks of cyclizing enzyme partially purified from porcine pituitary by DEAE-cellulose chromatography as described under "Materials and Methods."** Peak 1 represents DEAE fractions 18–21 (0.17 mg of protein/ml) and peak 2 represents fractions 27–31 (0.28 mg of protein/ml). Control incubations contained only the enzyme preparation and Gln-His-Pro-NH_2. Abbreviations: *Asc*, ascorbate; *NEM*, *N*-ethylmaleimide; *PMSF*, phenylmethylsulfonyl fluoride; *DDC*, diethyldithiocarbamate; *DTT*, dithiothreitol; *FAD*, flavine adenine dinucleotide; *6-MP*, 6-methylpterin; *6-MP(r)*, reduced 6-methylpterin; and *THB_4*, reduced tetrahydrobiopterin.

Several peptide analogs were tested at equimolar concentrations (0.2 mM) with the substrate Gln-His-Pro-NH_2 for inhibition of the two peaks of activity (Fig. 5). Little or no inhibition was observed by the presence of the amino acids Asn, Glu, and Gln, or by the dipeptides <Glu-Ala or Gln-Gly. Furthermore, neither cyclopentylamine (2 mM) or cyclopentanol (2 mM) inhibited the enzymatic reaction (data not included in Figure). These results suggest the possibility that the enzyme requires a minimum of two peptide bonds for recognition of substrate and that the enzyme has very low affinity for <Glu and related congeners. Interestingly, several of the amino acids and dipeptides appeared to enhance the activity of the enzyme, especially Peak 1. As yet the mechanism for this activation is undetermined, but is not due to immunologic cross-reactivity between inhibitor and TRH in the RIA.

Isoelectric focusing of fractions 25–42 pooled from the Sephadex G-100 chromatography of porcine pituitary also gave two major peaks of enzymatic activity, one with a pI of 5.7 and a second, a pI of 7.2, possibly also indicating microheterogeneity of the enzyme protein. Overall recovery from the agarose-Sephadex slabs, however, was less than 10%.

Enzyme partially purified by the same procedures from human B lymphocyte cells P3X63Ag8 (secreting) and P3X63Ag8 653 (nonsecreting) when chromatographed on DEAE-cellulose produced only one major peak of activity (0.013 nmol/h/μg, [substrate] = 0.2 mM) that eluted with 250 mM NaCl. Conversely, DEAE fractionation of neonatal rat brains also produced only one major peak of enzymatic activity, but which eluted with 100 mM NaCl (0.015 nmol/h/μg, [substrate] = 0.2 mM).

From these studies, it is likely that during the post-translational processing of precursors of many secretory proteins in the endoplasmic reticulum, Golgi, or secretory vesicle, "unmasking" of an N-terminal glutamine is a signal causing the enzymatic generation of a pyroglutamyl-peptide. Further support for this proposal is the observation that over 90% of the glutamine-peptide cyclase found in bovine adrenal medulla is contained within the soluble chromaffin vesicle fraction.[2] We conclude, therefore, that the cyclization of an N-terminal glutamine is not a random, spontaneous event, but an enzymatically driven reaction the control of which is biologically advantageous. Finally, if this enzyme is of importance in the post-translational processing of secretory peptides and proteins, then these results also suggest the presence of as yet undiscovered biologically active <Glu-peptides in adrenal medulla and pituitary.

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[2] E. DiLiberto, U. Viveros, J. S. Kizer, unpublished observations.

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